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Arabidopsis Yak1 protein (AtYak1) is a dual specificity protein kinase

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ABSTRACT

Yak1 is a member of dual-specificity Tyr phosphorylation-regulated kinases (DYRKs) that are evolutionarily conserved. The downstream targets of Yak1 and their functions are largely unknown. Here, a homologous protein AtYAK1 was identified in *Arabidopsis thaliana* and the phosphoprotein profiles of the wild type and an *atyak1* mutant were compared on two-dimensional gel following Pro-Q Diamond phosphoprotein gel staining. Annexin1, Annexin2 and RBD were phosphorylated at serine/threonine residues by the AtYak1 kinase. Annexin1, Annexin2 and Annexin4 were also phosphorylated at tyrosine residues. Our study demonstrated that AtYak1 is a dual specificity protein kinase in Arabidopsis that may regulate the phosphorylation status of the annexin family proteins.

1. Introduction

Phosphorylation is the most frequent type of protein post-translational modification, which is often associated with the molecular activation/de-activation of signal-transduction pathways [1]. In higher eukaryotes, an estimated one third of all proteins are regulated by phosphorylation by protein kinases (PKs). The genomes of plant species encode more than 1000 PKs [2]. However, the substrates of only a small fraction of these kinases are known [3].

Protein kinases are generally classified into serine (Ser)/threonine (Thr) kinases and tyrosine (Tyr) kinases, based on their substrate specificity. Several studies have shown that plants have dual protein kinases that are classified into a serine/threonine kinase family but catalyze phosphorylation on both serine/threonine and tyrosine residues [4,5], indicating that these plant kinases exhibit dual specificity. Several plants kinases have also been shown to autophosphorylate on Tyr, Ser and Thr *in vitro* [6]. In *Arabidopsis thaliana*, a few protein Tyr-specific phosphatases and 22 dual-specific phosphatases have been identified [1].

Yak1 is a member of Ser/Thr protein kinases family known as dual specificity of Tyr phosphorylation regulated kinases (DYRKs) in *Saccharomyces cerevisiae* [7]. In *S. cerevisiae*, Yak1 was originally identified as a negative regulator downstream of Ras/PKA signaling pathway. Yak1 is phosphorylated by PKA *in vitro* at several serine and threonine residues and regulated glucose starvation *in vivo*. However, the regulatory mechanisms of Yak1 activity and functions are largely unknown.

The present study was undertaken to identify proteins that are phosphorylated by Yak1 in *A. thaliana.* Of the seven candidate proteins selected by 2-DE analysis, Annexin1, Annexin2, Annexin4 and RNA binding family protein (RBD) were confirmed with phosphorylation assay and MS analysis. Annexin1, Annexin2 and RBD were phosphorylated at serine/threonine residues by AtYak1 kinase. Annexin1, Annexin2 and Annexin4 were additionally phosphorylated at tyrosine residues. Our data suggest that AtYak1 is dual specificity protein kinase in Arabidopsis.

Abbreviations: PK, protein kinase; RBD, RNA binding family protein; DYRKs, dual specificity of Tyr phosphorylation regulated kinases; 2-D PAGE, two dimensional polyacrylamide gel electrophoresis; GST, glutathione S-transferase

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2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis (*A. thaliana*) wild-type plants, T-DNA insertion mutant plants used in this work were of the Col-0 ecotype. The *atyak1-1* (SAIL_31_C10) and *atyak1-2* (SALK_0746) T-DNA insertion mutant alleles were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). Seeds were sterilized and germinated as described elsewhere [8]. Seven-day-old seedlings were harvested for total protein extraction.

2.2. Total protein extraction

Proteins were extracted from *atyak1-1* T-DNA mutant and wild-type plants by trichloroacetic acid (TCA)/acetone precipitation as described [9], with slight modifications. Briefly, 500 mg of tissue were ground to a fine powder in liquid nitrogen, suspended in 10% (w/v) TCA/acetone containing 0.06% (w/v) sodium sulfite and incubated overnight at −20 °C. The extract was centrifuged at 12,000×*g* for 20 min at 4 °C, washed three times in 80% (v/v) ice-cold acetone and air-dried for 10 min to remove residual acetone. Pellet was resuspended in rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM Tris–HCl (pH 8.0), 0.001% (v/v) tributylphosphine (TBP), vortexed overnight and centrifuged at 12,000×*g* for 20 min. Protein was quantified by Bradford assay.

2.3. 2-D PAGE and image analysis

For 2-D PAGE (two dimensional polyacrylamide gel electrophoresis) 100 μg of protein was adjusted to 125 μl with rehydration buffer supplemented with 1% (v/v) ASB-14, 0.2% (w/v) DTT and 0.5% (v/v) ampholyte. Proteins were focused using the IPGphor II (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) at 20 °C, 10,000 V. Strips were equilibrated in 6 M urea, 50 mM Tris–HCl (pH 8.8), 2% (w/v) SDS, 30% (v/v) glycerol containing 1% (w/v) DTT for 20 min followed by incubation in the same buffer containing 2.5% (w/v) iodoacetamide for another 20 min and then run in 12% SDS–PAGE. Phosphoproteins were stained using Pro-Q diamond phosphoprotein gel staining kit (Invitrogen Cat. No# MPP33000) following the manufacturer's instructions. Proteins were visualized using excitation at 532 nm with 560 nm longpass emission filter by Typhoon 9410 scanner (GE Healthcare). Image analysis was performed with Delta2D Software Version 4.3 (DECODON, www.decodon.com). Only proteins with significant and reproducible changes were considered to be differentially expressed.

2.4. In-gel trypsin digestion and MALDI-TOF analysis

Enzymatic digestion was performed as previously described [10]. Briefly, digestion was performed with 10 ng/ml of trypsin and 100 mM ammonium bicarbonate and incubated overnight at 37 °C. Following enzymatic digestion, the resultant peptides were extracted with 20 mM ammonium bicarbonate and twice with 5% formic acid in 50% acetonitrile and dried in SpeedVac. The peptides for LC–MS were suspended in 5% acetonitrile and 0.1% formic acid, and those for MALDI-TOF were suspended in 5% acetonitrile and 0.1% trifluoroacetic acid. Peptide masses were measured on a MALDI-TOF (maXis UHR-TOF, Bruker) [11]. Peptide mass fingerprint data were matched to the TAIR10 Arabidopsis protein sequence database by using MASCOT and scaffold software.

2.5. Expression and purification of recombinant protein

GST fusion protein, pGEX6p-AtYak1, pGEX6p-AnnAt1, pGEX6p-AnnAt2, pGEX6p-AnnAt4, pGEX6p-CAB3, pGEX6p-RCA, and pGEX6p-RBD were generated using primers sets listed in Table S1. Protein expression and purification was performed as described [12] with modifications. Briefly, 50 ml of bacterium cell culture were harvested by centrifugation, washed once with 20 ml of ice-cold STE [10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA] and resuspended by repeated pipetting in 5 ml of STE containing 100 μg/ml of lysozyme. After incubation on ice for 20 min, dithiothreitol (DTT) was added to a final concentration of 5 mM. Protease inhibitors were also added. Bacteria were lysed by adding 1.5% N-lauroylsarcosine (Sarkosyl). The cells were then vortexed for 5 s and sonicated on ice for 1 min. Thereafter, the lysate was centrifuged for 10 min at full speed at 4 °C. The supernatant was collected and mixed with 2% Triton X-100. Then, 200 μl of washed glutathione agarose bead suspension (50% v/v in PBS) was added and the lysate was incubated at 4 °C for 15 min. The beads were washed 6 times with ice-cold PBS [8.4 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, pH 7.4, 150 mM NaCl] by repeated centrifugation and were resuspended in elution buffer [75 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM reduced glutathione, 5 mM DTT]. For storage, glycerol was added to a final concentration of 10% (v/v) and the protein solution was kept at −80 °C.

2.6. In vitro phosphorylation assays

AtYak1 kinase activity assays were performed with 20 ng of GST-AtYak1 purified protein and 1 μg of target protein in a 50 μl reaction volume. Assay buffer contained 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 2 mM EGTA, and 2.5 mM $CaCl_2$ with 60 mM ATP as a phosphate donor. Kinase assay components were assembled in each tube on ice. After being transferred to 30 °C, ATP was added and the mixture was incubated for 30 min. Reactions were stopped by adding 10 μl of SDS sample loading buffer. Aliquots of 30 μl of each reaction mixture were resolved in 12% SDS–PAGE gel and stained with Coomassie Blue.

2.7. Mass spectrometry

The peptides were fractionated by C_{18} RP chromatography and analyzed online by mass spectrometry (LC–MS/MS). Mass spectrometry analysis was carried out using LTQ Orbitrap Velos (Thermo-Scientific) mass spectrometer connected to a nano-LC system (Proxeon). Peptides (5 μl injection) were loaded onto a C_{18} trap column (Michrom), washed, followed by elution at 400 μl/min flow rate via on line analytical column (Michrom 3 μM, 200 Å, Magic C18AQ, 0.1 × 150 mm) connected to a nano-spray source (Proxeon). The peptide elution from C_{18} column was achieved by a 25 min linear gradient of 5–35% (v/v) acetonitrile in 0.1% (v/v) formic acid, and a 5 min linear gradient of 35–80% (v/v) acetonitrile in 0.1% (v/v) formic acid, followed by 10 min isocratic flow of 80% (v/v) acetonitrile in 0.1% (v/v) formic acid. The eluted peptides were analyzed online by electrospray ionization tandem mass spectrometry, Xcalibur (v2.1) software (Thermo-Scientific) was used to collect mass spectrometry data, and mass range for the MS survey scans was set at *m*/*z* 350–1600. The *m*/*z* value of ions during first full scan was determined at high accuracy using Orbitrap mass analyzer. Each MS scan was followed by 10 MS/MS scans, and the data was collected using LTQ-linear ion trap. MS3 scan was selectively triggered upon neutral loss ion (32.7, 49, 80, and 98) detection. We used a dynamic exclusion method where a specific ion was sequenced twice at maximum and excluded from the list for 45 s.

Table 1
Functional annotation of classified phosphoproteins.

Spot No.[a]	Protein name[b]	Accession No.	Match MW (kD)/pI[c]	Apparent MW (kD)/pI[d]	Identification probability[e] (%)	Percent covered[f] (%)	Fold change	
5	GTP binding elongation factor Tu family protein	AT1G07920.1	49.5/9.6	41.5/4.3	100.00	23.50	−1.51	
6	Tubulin beta-7 chain (TUB7)	AT2G29550.1	50.7/4.5	32.0/4.3	100.00	18.90	−0.84	Salt stress response
8	Glyceraldehyde-3-phosphate dehydrogenase of plastid 2 (GAPCP-2)	AT1G16300.1	44.8/8.9	25.0/4.5	100.00	20.50	−0.38	Root development
13	Chloroplast RNA-binding protein 29 (CP29)	AT3G53460.1	38.4/5.2	30.0/4.8	100.00	19.90	3.58	Cold stress response
14	Pyrophosphorylase 6 (AtPPa6)	AT5G09650.1	33.8/5.8	30.0/4.9	100.00	17.30	−5.42	Cold stress, salt stress response
15	RNA-binding (RRM/RBD/RNP motifs) family protein	AT2G37220.1	30.7/4.7	24.5/4.6	99.80	11.40	−0.50	Light reaction, ABA & cold response
16	RNA-binding (RRM/RBD/RNP motifs) family protein	AT2G37220.1	30.7/4.7	24.5/4.6	100.00	17.60	−2.16	Light reaction, ABA & cold response
20	Ribulose-bisphosphate carboxylases (RBCL)	ATCG00490.1	52.9/6.2	60.0/5.0	100.00	34.40	−9.15	ABA response
20	Trigger factor type chaperone family protein	AT5G55220.1	61.7/5.0	61.7/5.0	100.00	25.60	9.15	
21	Chlorophyll A/B binding protein 3 (CAB3, AB180)	AT1G29910.1	28.2/5.0	40.0/4.8	99.80	3.75	−7.99	Photosynthesis
22	Chlorophyll A/B binding protein 3 (CAB3, AB180)	AT1G29910.1	28.2/5.0	40.0/4.8	100.00	8.99	−55.80	
23	FtsH extracellular protease family (VAR1, FTSH5)	AT5G42270.1	75.2/5.1	72.0/5.2	100.00	11.80	−2.01	
25	Phosphoribulokinase (PRK)	AT1G32060.1	44.4/5.9	44.0/5.2	100.00	10.10	−3.09	
26	Annexin1 (ANNAT1)	AT1G35720.1	36.2/5.0	42.0/5.2	100.00	16.10	−1.32	Cold, heat, osmotic, salt and ABA response
27	Ribosomal protein L10 family protein	AT3G11250.1	34.4/4.7	40.0/5.1	100.00	8.36	−2.78	Salt stress response
28	Thioredoxin superfamily protein	AT3G52960.1	24.7/9.5	15.0/5.0	100.00	13.20	2.39	
30	Ureidoglycolate amidohydrolase (ATAAH-2, UAH)	AT5G43600.1	51.5/5.3	60.0/5.5	99.80	3.99	2.31	
31	Insulinase (Peptidase family M16) protein	AT1G51980.1	54.4/6.2	55.0/5.5	100.00	14.50	−0.96	Salt stress response
34	Rubisco activase (RCA)	AT2G39730.1	51.9/5.9	54.0/5.1	100.00	7.17	−0.52	Cold, light & JA response

[a] Number of spot.
[b] Identified protein name.
[c] Predicted molecular weight (MW) and pI of match peptide sequence.
[d] Observed molecular weight (MW) and pI of spot from the gel.
[e] Percentage of protein identification probability.
[f] Percentage of predicted protein sequence covered by matched peptides.

The RAW mass spectrometry data files were converted to mgf format using Proteome Discoverer 1.2 (Thermo-Scientific), and the MS/MS data sets were compared with the TAIR10 Arabidopsis protein sequence database using Mascot search engine (v2.3, Matrix Science).

3. Results

3.1. AtYak1 T-DNA insertion line genotyping

We obtained two alleles of *atyak1* T-DNA insertion mutants from the Arabidopsis Biological Resource Center. The T-DNA was inserted in the first intron in the *atyak1-1* (SAIL_31_C10) mutant and in the ninth intron in the *atyak1-2* (SALK_074646) mutant (Fig. 1A). RT-PCR screening of these homozygous lines revealed complete knockout of AtYak1 in the *atyak1-1* mutant yet the *AtYAK1* transcripts were still observed in *atyak1-2* (Fig. 1B). We checked the phenotype of *atyak1-1*, *atyak1-2* and wild-type plants under normal growth conditions. *atyak1-1* plants had smaller and fewer leaves and displayed reduced biomass than the wild-type plants, whereas there was no difference in growth and biomass between *atyak1-2* plants and wild-type plants (unpublished data). The results show that *atyak1-1* was a null allele and thus was used in the current study.

3.2. Classification of identified phosphorylated proteins

Total proteins isolated from wild-type and *atyak1-1* mutant seedlings were resolved by 2D gel electrophoresis and phosphoproteins were identified by staining using Pro-Q diamond phosphoprotein gel staining kit. In either the mutant or wild-type samples, about 150 protein spots were found to distribute evenly between pH 4 and 7 and the molecular masses of these proteins range from 10 to 80 kD. To classify these phosphorylated proteins, we used Classification Superviewer. Proteins were assigned to 13 classes based on their domain composition or functional description in the literature (Fig. 2). Phosphorylated proteins involved in metabolic and cellular processes were the most abundant, whereas proteins involved in developmental processes, transport and other signaling processes were the least abundant. Between the *atyak1-1*

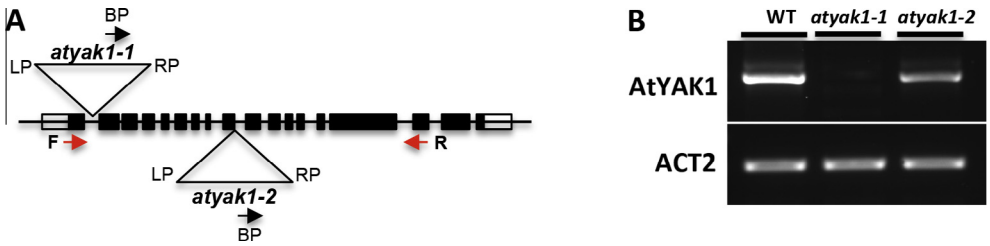

Fig. 1. Genotyping and gene expression analyses of the AtYak1 T-DNA insertion mutants. (A) Scheme of *AtYak1* gene. The triangle indicates the position of the T-DNA insertion. Genomic AtYak1 DNA sequence is represented by exons (solid black bars), introns (thin black lines) and UTRs (white bars). Homozygous lines were genotyped with LP, RP and BP primers. (B) RT-PCR analysis of *AtYak1* expression in wild-type and homozygous *atyak1-1* and *atyak1-2* mutant plants. Red arrows (in (A)) indicate positions of primers used in RT-PCR. *ACT2* was used as a control.

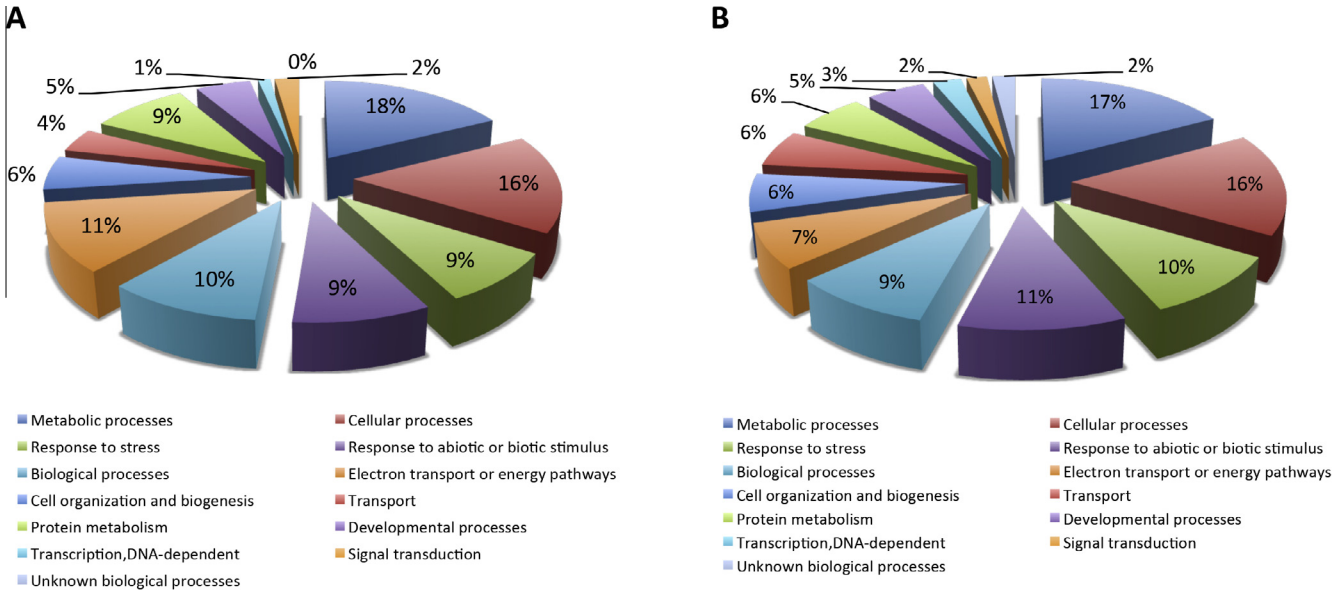

Fig. 2. Functional classification of the identified phosphorylated proteins. (A) The pie chart shows the identified phosphoprotein of wild-type Col-0. One hundred and fifteen peptides were identified. (B) Identified phosphoprotein of *atyak1-1* mutant. One hundred and fifty peptides were identified. Identified proteins were grouped according to functional classification in Classification SuperViewer (http://bar.utoronto.ca/ntools/cgi-binntools_classification_superviewer.cgi).

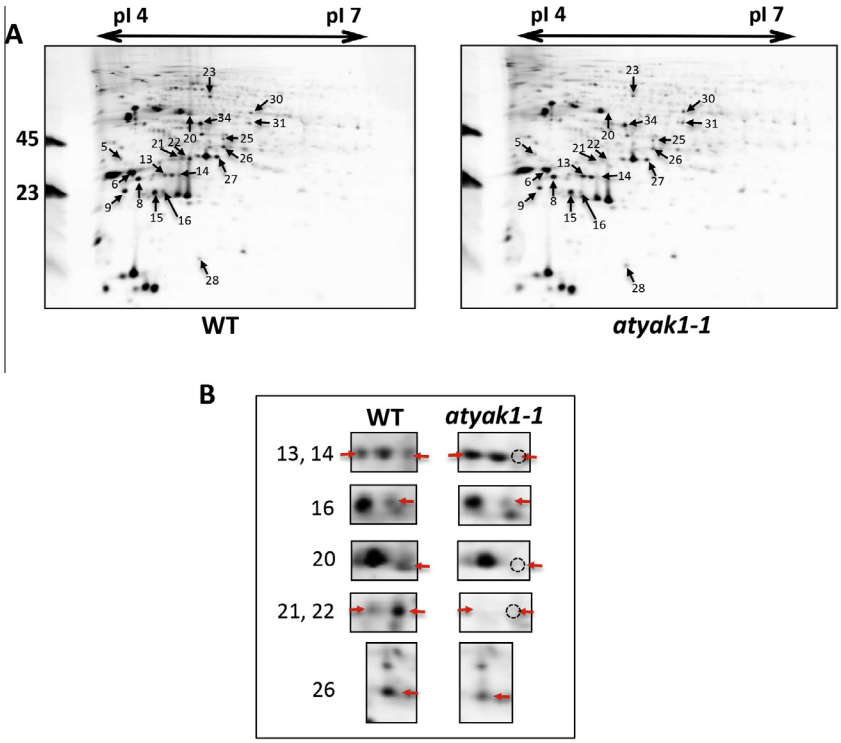

Fig. 3. Comparison of phosphorylated proteins between wild-type and *atyak1-1* mutant plants. The gels were stained with Pro-Q Diamond phosphorylation stain kit. (A) Total phosphorylated proteins in the range of pH 4–7. Total phosphoproteins in the wild-type (left) and *atyak1-1* mutant (right). Protein spots identified by MALDI-TOF MS are numbered and listed in Table 1. (B) Selected phosphoproteins with changed expression levels. Circles indicate absence of phosphoprotein in the *atyak1-1* mutant.

mutant and the wild-type plants, three categories of proteins were identified: proteins that show the same phosphopeptide abundance in both the wild-type and the *atyak1-1* mutant, proteins that show decreased phosphopeptide abundance in the *atyak1-1* mutant plants compared with the wild-type, and proteins that show increased phosphopeptide abundance in the *atyak1-1* mutant compared with the wild-type. For example, phosphopeptide proteins involved in metabolic and cellular processes showed the same abundance in both the *atyak1-1* mutant and the wild-type plants. Phosphorylated proteins involved in electron transport or energy pathways showed decreased abundance in the *atyak1-1* mutant compared with wild-type, whereas those involved in biotic

and abiotic stress response showed increased abundance in the *atyak1-1* mutant compared to wild-type plants (Fig. 2). Full lists of the identified proteins are provided in Table S2.

3.3. Identification of phosphorylated proteins

Among the phosphoproteins separated on the 2-D gel, we selected 50 spots based on differences in their intensity between the wild-type and the mutant and revealed the identities of these proteins with MALDI-TOF MS (Fig. 3A and Table 1). To analyze the changes in spot intensity between the wild-type and *atyak1-1* mutant, samples were quantified (Section 2.3). Six spots (numbers 13, 14, 16, 20, 21, 22 and 26) representing protein p13, p14, p16, p20, p21 p22 and p26, respectively, were found to be differentially expressed or phosphorylated between the wild-type and the mutant (Fig. 3B). As compared to the wild-type, the level of expression or phosphorylation of p13 increased, whereas that of p14, p16, p20, p21 p22 and p26 seemed to decrease or not detected in the *atyak1-1* mutant.

Among these proteins, p26 (spot number 26) was identified as Annexin1 (AnnAt1), which has been implicated in responses to heat, cold, salt, osmotic stress and abscisic acid (ABA) in plants [12]. The intensity of the p26 spot was higher in the wild-type than in the *atyak1-1* mutant (Fig. 3A). Protein p26 migrated with a molecular mass of 42.0 kD (Table 1), which is larger than the standard molecular size of AnnAt1 (36.2 kD). This difference may be because of the different developmental stages or growth conditions of the plants under investigation [13]. The apparent pI value of p26 is 5.2, similar to the standard value of 5.0. Phosphoproteins p21 and p22 (spot numbers 21 and 22) were identified as chlorophyll A/B binding protein 3 (CAB3, AB180) that functions in photosynthesis. p21 and p22 spots were only observed in the wild-type but not in the *atyak1-1* mutant (Table 1), suggesting that their expression and/or phosphorylation is dependent on AtYak1. These proteins and their annotations are listed in Table 1.

3.4. In vitro phosphorylation assay

Among the phosphorylated proteins identified, we selected AnnAt1, AnnAt2, AnnAt4, CAB3, RBD and RCA as target proteins for further characterization. We purified six GST fused target proteins from the *Escherichia coli* BL21. For *in vitro* phosphorylation, target and substrate protein combination and enzyme reactions are shown in Supplementary Fig. 1. After enzyme reaction, each protein band was isolated for phosphoprotein detection by LC–MS/MS (LTQ Orbitrap). Our *in vitro* phosphorylation assay analysis shows that all 3 members of annexin family, AnnAt1, AnnAt2 and AnnAt4, were phosphorylated by AtYak1 kinase. And AtYak1 kinase was phosphorylated by itself. On the other hand, CAB3 and RCA were not phosphorylated by AtYak1 kinase (Supplementary Fig. 1).

3.5. MS identification of phosphorylation sites

To identify the sites where the AtYak1 kinase and target proteins, annexins and RBD, were phosphorylated, we conducted LC–MS/MS analysis. Annotated LC–MS/MS spectra showing peptide fragmentation and predicted phosphorylation sites are presented in Figs. 4 and 5, and in Table 2. Fragmentation patterns of the doubly charged ions 448.76 *m*/*z* from the AtYak1 and 805.86 *m*/*z* from the RBD peptide digests (Fig. 4A and B) resulted in the match of the sequence of peptide fragment 895.51 Da of

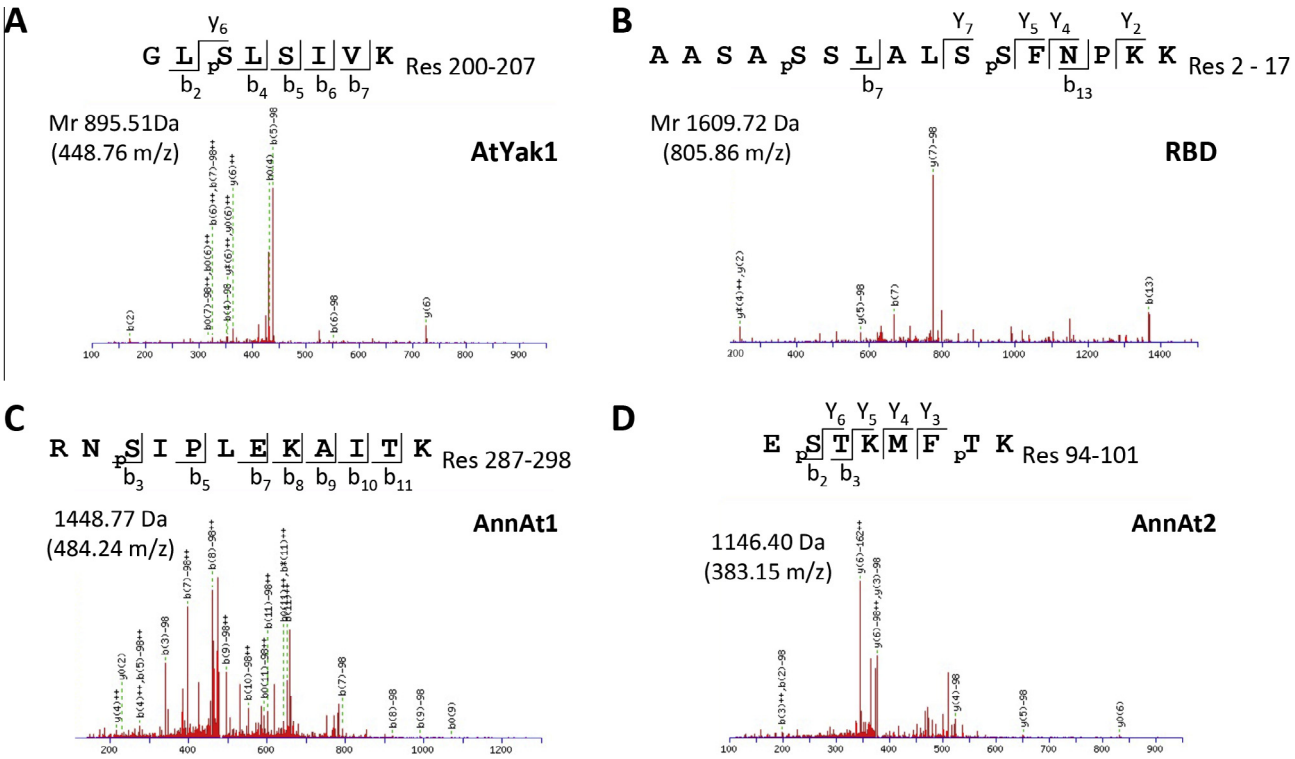

Fig. 4. Serine/threonine phosphorylation site identification of AtYak1, RBD, AnnAt1 and AnnAt2 by LC–MS/MS. Fragmentation patterns of the doubly charged ions 448.76 *m*/*z* from the AtYak1. The amino acid sequence of the peptide fragments 895.51 Da of AtYak1 is shown with the phosphorylation site of Ser-202 by autophosphorylation (A) and 805.86 *m*/*z* from the RBD peptide digests. The amino acid sequence of the peptide fragments 1609.72 Da of RBD is shown with the phosphorylation sites of Ser-6 and Ser-12 by AtYak1 kinase (B). Fragmentation pattern of the doubly charged ions 484.24 *m*/*z* from AnnAt1 (C). Fragmentation pattern of the doubly charged ions 383.15 *m*/*z* from AnnAt2 (D). The amino acid sequences of the peptide fragments 1448.77 and 1146.40 Da are shown with the phosphorylation site of Ser-289 from AnnAt1 and Ser-95 and Thr-100 from AnnAt2 by AtYak1 kinase.

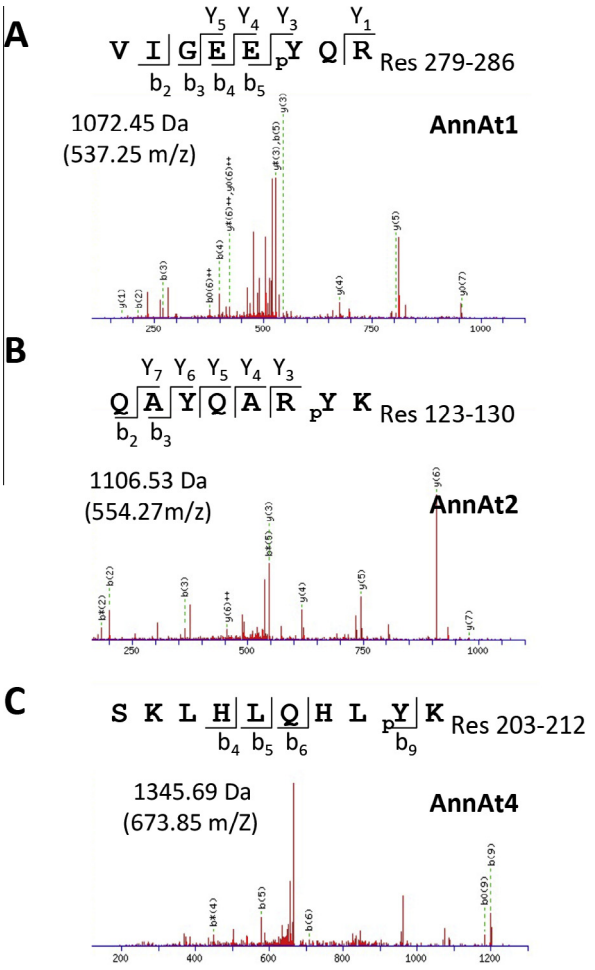

Fig. 5. Tyrosine phosphorylation site identification of AnnAt1 and AnnAt2 by LC–MS/MS. Fragmentation patterns of the doubly charged ions 537.25 *m*/*z* from the AnnAt1. The amino acid sequence of the peptide fragments 1072.45 Da of AnnAt1 is shown with the phosphorylation site of Tyr-284 (A) and 554.27 *m*/*z* from the AnnAt2 peptide digests. The amino acid sequence of the peptide fragments 1106.53 Da of AnnAt2 is shown with the phosphorylation site of Tyr-126 (B). Fragmentation pattern of the doubly charged ions 673.85 *m*/*z* from AnnAt4. The amino acid sequences of the peptide fragments 1345.69 Da are shown with the phosphorylation site of Tyr-211 from AnnAt4 (C). Annexin family (AnnAt1, AnnAt2 and AnnAt4) shown tyrosine phosphorylation by AtYak1 kinase.

AtYak1 and 1609.72 Da of RBD. The phosphorylation site of AtYak1 was deduced to be located at the Ser-202 by autophosphorylation (Fig. 4A and Table 2). Interestingly, this autophosphorylation site differs from yeast where autophosphorylation was observed at tyrosine residues in the activation loop among the DYRKs [14]. The phosphorylation sites of RBD were located at Ser-6 and Ser-12 (Fig. 4B and Table 2). The identification of the phosphorylation sites in the AnnAt1 and AnnAt2 was performed similarly. Fragmentation pattern of the doubly charged ions 484.24 *m*/*z* from AnnAt1 and 382.15 *m*/*z* from AnnAt2 matched the sequence of peptide fragment 1448.77 Da of AnnAt1 (Fig. 4C) and 1146.40 Da of AnnAt2 (Fig. 4D). The amino acid sequences of the peptide fragment 1448.77 Da showed that the phosphorylation site was located at Ser-289 (Fig. 4C and Table 2). The phosphorylation sites of AnnAt2 were identified at Ser-95 and Thr-100 (Fig. 4D and Table 2).

The Annexin family members (AnnAt1, AnnAt2 and AnnAt4) also phosphorylated at tyrosine by AtYak1 kinase. Fragmentation patterns of the doubly charged ions 537.25 *m*/*z* from the AnnAt1, 554.27 *m*/*z* from the AnnAt2 and 673.85 *m*/*z* from AnnAt4 matched the sequence of the peptide fragments 1072.45 Da of AnnAt1 (Fig. 5A and Table 2), 1106.53 of AnnAt2 (Fig. 5B) and 1345.69 of AnnAt4 (Fig. 5C). The phosphorylation site of AnnAt1, AnnAt2 and AnnAt4 was located at Tyr-284, Tyr-126 and Tyr-211, respectively (Fig. 5A–C and Table 2).

4. Discussion

The aim of this study was to identify components of the AtYAK1 signaling pathway in Arabidopsis by examining the phosphorylation status of the proteomes of wild-type and the *atyak1-1* mutant plants. In this study, we used two-dimensional gel electrophoresis and LC–MS/MS to identify a number of candidates that showed altered phosphoprotein/peptide abundance in the *atyak1-1* mutant. Several protein spots were identified that showed altered phosphopeptide abundance in the *atyak1-1* mutant. Through proteomic analysis, we identified and selected Annexins for further characterization. Annexins are a family of Ca^{2+}-dependent membrane binding proteins that exist in almost all species from fungi to humans [15]. These proteins have been shown to play important roles in various cellular processes such as membrane trafficking and organization, regulation of ion channel activity, inflammatory response, mitotic signaling, and phospholipid metabolism [16]. Our results demonstrate that the abundance or phosphorylation of Annexin family proteins was lower in the *atyak1-1* mutant (Fig. 3B). *In vitro* experiments suggest that AtYak1 may directly phosphorylate annexin proteins in Arabidopsis.

Although in most plant species no more than two annexin genes have been reported, in Arabidopsis, seven distinct Annexin genes have been identified, which are AnnAt1–AnnAt7 [17], with AnnAt4 being the most divergent. AnnAt1 and AnnAt4 have been suggested to play important roles in osmotic and ABA signaling [13]. Here, we identified three Annexins, AnnAt1, AnnAt2 and AnnAt4, which were phosphorylated at serine/threonine and tyrosine residues by AtYak1 kinase.

Yak1 belongs to a dual-specificity tyrosine-regulated protein kinase (DYRK) family that autophosphorylate an essential tyrosine

Table 2
Phosphorylation sites identified within AtYak1 and target protein *in vitro* phosphorylation assay by LC–MS/MS.

Protein	Peptide and phosphorylation site(s)	Locus	Residues	Describe*
AtYak1	GL**pS**LSIVK	At5g35980.1	220–227	Protein kinase, autophosphorylation
AnnAt1	YIGEE**pY**QR	At1g35720.1	279–286	Annexin gene family, Tyr
AnnAt1	RN**pS**IPLEKAITK	At1g35720.1	287–298	Annexin gene family, Ser/Thr
AnnAt1*	RWTS**pS**NQVLMEVAC**pT**RTSTQLLHAR	At1g35720.1	98–122	Annexin gene family, Ser/Thr
AnnAt1*	LLVSLVT**pSpY**RYEGDEVNMTLAK	At1g35720.1	148–169	Annexin gene family, Ser/Thr, Tyr
AnnAt2	QAYQAR**pY**K	At5g65020.1	123–130	Annexin gene family, Tyr
AnnAt2	E**pS**TKMF**pT**K	At5g65020.1	94–101	Annexin gene family, Ser/Thr
AnnAt4	SKLHLQHL**pY**K	At2g38750.1	203–212	Annexin gene family, Tyr
AnnAt4*	ADH**pS**DEMNEIK	At2g38750.1	274–284	Annexin gene family, Ser/Thr
RBD	AASA**pS**SLALS**pS**FNPKK	At2g37220.1	2–17	Chloroplast RNA binding protein, Ser/Thr

pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.
* Ions score below 10.

in their activation loop, but phosphorylate their substrate at several serine and threonine residues [14] and regulate glucose starvation *in vivo*. Studies have shown that Yak1 acts downstream of the PKA pathway [18] and that it controls transcriptional factors involved in stress response [19] and ribosomal biogenesis [18]. In previous studies, Yak1 was shown to phosphorylate casein [20] and autophosphorylate at tyrosine residues when expressed in yeast [14]. In this study, we showed that AtYak1 protein kinase phosphorylates its substrates on Ser/Thr residues and undergoes Ser-202 autophosphorylation, indicating that Yak1 is a dual specificity protein kinase. In yeast, autophosphorylation was observed at tyrosine residues in the activation loop among the DYRKs [14]. In our experiment, autophosphorylation was detected at the Ser-202 residue. This difference may be due to the different studied organisms or incomplete peptide sequencing of AtYak1.

In conclusion, this study demonstrates that AtYak1 is a dual specificity protein kinase in Arabidopsis, which phosphorylates substrate proteins on Ser/Thr and Tyr residues. We are currently undertaking studies to find out the role of AtYak1 in stress response in Arabidopsis.

Conflict of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.09.025.

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